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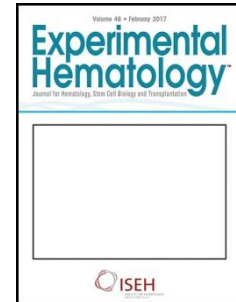
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Title Page**A comparison of intrauterine haemopoietic cell transplantation and lentiviral gene transfer for the correction of severe β -thalassaemia in a HbbTh3/+ murine model.**

Niraja M. Dighe^{a*}, Kang Wei Tan^{a*}, Lay Geok Tan^a, Steven S.W. Shaw^{b,c}, Suzanne M.K. Buckley^d, Dedy Sandikin^a, Nuryanti Johana^e, Yi-Wan Tan^e, Arijit Biswas^a, Mahesh Choolani^a, Simon N. Waddington^{d,f}, Michael N. Antoniou^g, Jerry K.Y. Chan^{e,h}, and Citra N.Z. Mattar^{a**}.

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Running head: Intrauterine therapy in a thalassaemia mouse model

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Non-standard abbreviations

IUGT – Intrauterine gene transfer

IUHCT – Intrauterine haemopoietic cell transplantation

LV – lentiviral vector

SCD – sickle cell disease

HSCT – haemopoietic stem cell transplantation

AAALAC - Association for Assessment and Accreditation of Laboratory Animal Care International

HET – heterozygous hybrid pup (HbbTh3/+) from crossing CD1 females with HbbTh3/+ males

WT – wild type

MNC – mononuclear cell

Highlights

- **HbbTh3/+ mouse is a good model of severe thalassaemia for in-utero therapy**
- **Better chimerism after in-utero and postnatal transplantation with immunosuppression**
- **In-utero gene therapy produced partial haematological correction but not full rescue**
- **Both strategies need further optimisation to overcome hostile microenvironment**

Abstract

Major haemoglobinopathies place tremendous strain on global resources. Intrauterine haemopoietic cell (IUHCT) and gene (IUGT) therapies can potentially reduce perinatal morbidities with greater efficacy than postnatal therapy alone. We performed both procedures in the thalassaemic HbbTh3/+ murine model. Intraperitoneal delivery of coisogenic cells at E13-14 produced dose-dependent chimerism. High-dose adult bone marrow (BM) cells maintained 0.2-3.1% chimerism over ~24 weeks and treated heterozygotes demonstrated higher chimerism than wild-type pups (1.6 vs. 0.7%). Fetal liver cells produced higher chimerism compared to adult BM when transplanted at the same doses, maintaining 1.8-2.4% chimerism over ~32 weeks. We boosted transplanted mice postnatally with adult BM cells following busulfan conditioning. Engraftment was maintained at >1% only in recipients which were chimeric prior to boosting. IUHCT-treated non-chimeras and non-IUHCT mice showed micro- or no chimerism. Additional fludarabine treatment produced higher chimerism than busulfan alone. Engraftment was more effective following higher starting chimerism prior to boosting and in heterozygotes. Chimeric heterozygotes expressed 2.2-15.1% donor cells with eventual decline at 24 weeks (vs. <1% in non-chimeras) and demonstrated improved haematological indices and smaller spleens compared to untreated heterozygotes. Intravenous delivery of GLOBE lentiviral-vector expressing HBB (human β -globin) resulted in vector concentration of 0.001-0.6 copies/cell. Most haematological indices were higher in treated than untreated heterozygotes including haemoglobin and mean corpuscular volume, though still lower than in wild-types. Thus both direct IUGT and IUHCT strategies can be used to achieve haematological improvement but

require further dose optimisation. IUHCT will be useful combined with postnatal transplantation to further enhance engraftment.

Word count 250

Key words Thalassaemia, intrauterine gene therapy, haemopoietic stem cell, intrauterine cell therapy, postnatal transplantation, fludarabine, HbbTh3/+ mouse, GLOBE vector.

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Introduction

The haemoglobinopathies are the most prevalent monogenetic disorders generating substantial medical and socioeconomic burden worldwide [1]. α -thalassaemia major is perinatally lethal and necessitates effective intrauterine intervention to avoid the complications of chronic severe hypoxia evident in transfusion-dependent survivors [2]. β -thalassaemia major and sickle cell disease (SCD) demand substantial resources to prevent permanent organ failure [3]. Much of the disease burden arises from suboptimal treatment [4]. Curative postnatal allogeneic haemopoietic stem cell transplantation (HSCT) is available to one third of individuals with thalassaemia and requires bone marrow (BM) conditioning, risking well-documented complications [5]. Due to the projected expansion of at-risk populations there is an urgent need to formulate an early-intervention strategy that is effective and safe [6]. While α -thalassaemia major clearly requires an intrauterine remedy given the early fatality, fetal treatment of β -haemoglobinopathies is debatable as clinical manifestations only arise in infancy. However, acknowledging the risks of conventional treatment and the therapeutic advantage of youth, a strong argument can be made for fetal therapy in which the goal is reduction of disease burden [7, 8]. Potential benefits of intrauterine cell and gene therapy for these and other genetic disorders are widely described [9, 10]. Notable advantages of intrauterine haemopoietic cell transplantation (IUHCT) include the high donor cell:fetal mass ratio (dose-dependent response), immune-naïveté (donor cell tolerance) and diminished host competition for available haematopoietic niches [11]. Advantages of intrauterine gene transfer (IUGT) include the greater transducibility of fetal target cells and lower risk of immune-mediated clearance [10]. Potential correction of these conditions well before irreversible end-organ damage and avoidance of treatment-related morbidity underscores the expectation that intrauterine therapies will benefit both α - and β -thalassaemia major, similar to treatment of congenital immunodeficiency syndromes and osteogenesis imperfecta [2, 12].

Despite its promise, IUHCT has been largely disappointing in most monogenetic conditions due to host immune and competitive barriers [13]. In mice, achieving sustained engraftment within a competent host immune system requires a minimum initial donor cell chimerism of 1.8% [14]. Although higher engraftment has been achieved in animal models, therapeutic engraftment has been difficult to replicate in humans [15]. The unique micro-environment in the BM of thalassaemic individuals and the lack of a competitive advantage for donor cells suggests that a strategy more complex than a single IUHCT may be needed to reach therapeutic effect [16], such as [transplanting high-dose maternal donor cells within the optimal gestational window and T-cell manipulation of the donor inoculum](#) [17-19]. The [alternative approach of *in-vivo* intrauterine gene transfer \(IUGT\)](#) has been utilised in a murine α -thalassaemia model to achieve erythroid-specific α -globin expression lasting seven months [20]. In adult individuals with β -thalassaemia *ex-vivo* gene therapy has met with reasonable success; this approach is impractical in the fetus as it necessitates multiple invasive procedures [21]. IUGT may present an effective way to target fetal haematopoietic progenitors and has demonstrated success in treating other models of monogenetic disease [9]. HIV-1-based integrating lentiviral vectors (LV) are valuable in the treatment of haemoglobinopathies as they transduce quiescent haematopoietic stem cells (HSC), are less mutagenic than γ -oncoretroviruses and are becoming safer and more efficient for clinical use through improved design [21, 22]. To study and compare the outcomes of IUHCT and IUGT we utilised the HbbTh3/+ murine model in which surviving heterozygotes clinically represent severe β -thalassaemia intermedia while non-surviving homozygotes represent α -thalassaemia major [23]. We examined the additive effect of postnatal transplantation following IUHCT and the efficacy of a single intrauterine injection of LV-MA821 (GLOBE) expressing a human β -globin (*HBB*) transgene [24].

Materials and Methods

Animal experiments

Animal experiments were performed at the National University of Singapore (NUS), an AAALAC-accredited institution and followed guidelines described in the NIH Guide for the Care and Use of Laboratory Animals under the NUS Institutional Animal Care and Use Committee (IACUC) and the Office of Safety, Health and Environment (OSHE). B6.129P2-Hbb-b1^{tm1Unc}Hbb-b2^{tm1Unc}/J mice ("HbbTh3/+") and C57BL/6.CD45.1 ("B6") were purchased from the Jackson Laboratories (Bar Harbor, ME) while CrI:CD1[ICR] females ("CD1") were purchased from Charles River Laboratories (Wilmington, MA). C57BL/6-Tg[UBC-GFP]30Scha/J.CD45.2 mice ("B6-GFP") used as BM donors at 6-8 weeks were kindly donated by F. Ginhoux (Singapore Immunology Network, A*STAR, Singapore). We mated HbbTh3/+ males and HbbTh3/+ or B6 females for IUHCT performed intraperitoneally (IP) at E13-14, and HbbTh3/+ males and CD1 females for IUGT performed intravenously (IV) at E15-16. The day of plug observation was designated E0.5 (Supplemental Figure S1). Animals were sacrificed with inhalational CO₂ or cervical dislocation for terminal harvests.

Murine BM and fetal liver donor cell preparation

BM cells and fetal liver (FL) donor cells were harvested from long bones of B6-GFP adults and E13-14 fetuses respectively. Cells collected in phosphate buffered saline (PBS; Gibco, Grand Island, NY, USA) containing 2mM ethylenediaminetetraacetic acid (EDTA, Sigma) at pH7.2 were processed into single-cell suspensions by passage through a 22-gauge needle, and centrifuged over 15ml of Ficoll-Histopaque 1077 (Sigma, USA) as described [25]. Mononuclear cells (MNC) were harvested from the interphase layer, washed in PBS, frozen and thawed in batches for IUHCT. Donor MNC were consistently >95% for GFP, ~70% negative for lineage markers (Lin⁻) and ~20-40% cKit and Sca1 positive, thus we used the whole MNC component without further enrichment to prevent potential loss of proliferating long-term repopulating HSC [26]. Viable MNC confirmed by trypan blue exclusion were T-cell depleted with FITC anti-mouse CD3-antibody, incubated with anti-FITC microbeads and

passaged through magnetic columns (Mouse CD3 Depletion Kit, Miltenyi Biotec, Singapore)[27]. Flow cytometry (FACS) confirmed a CD3+ component <0.5% of the final inoculum. Final cell concentrations were prepared according to live cell counts. CD26 inhibition was performed with Diprotin A (Peptides International, Louisville, KY) prior to transplantation as described [28].

Lentiviral vector preparation

GLOBE is a self-inactivating HIV-based LV encoding mini-*HBB*, linked to the HS2 and HS3 elements of the *HBB* locus control region (LCR) [29]. LV stocks were generated by triple plasmid co-transfection of human embryonic kidney 293 (HEK293T) cells, using the packaging plasmid pCMV δ R8.2 (9.75 μ g), the envelope plasmid pMD2.G (5.25 μ g) and transfer vector plasmid MA821 (15 μ g for transfection of a single 75cm² flask) with a Calcium Phosphate Transfection Kit (Invitrogen, USA) as described [24, 30]. Briefly, we collected medium after 48 and 72 hours of transfection, concentrated vector particles by centrifugation (90,000g, 140 minutes, 4°C), re-suspended pellets in sterile PBS with 1% bovine serum albumin and quantified titers by quantitative PCR (qPCR), using forward primer 5'-TGAAAGCGAAAGGGAAACCA-3' and reverse primer 5'-CCGTGCGCGCTTCAG-3', specific for the rev-response element (RRE) region [24].

Intrauterine cell and vector administration

All procedures were performed under isoflurane anaesthesia with dams given caprofen and enrofloxacin (at 0.1ml/10g body-weight) before midline laparotomies at which uterine horns exteriorized and injections administered with a 34-gauge Hamilton needle (Bonaduz, Switzerland) under the stereomicroscope. For IUHCT, murine BM GFP+ MNC (B6-GFP) were delivered IP in 10 μ L, at 2E+6 (low-dose, BM^{LD}) or 5E+6 (high-dose, BM^{HD}) cells/fetus. FL cells were injected IP at 2E+6 (FL^{LD}) or 5E+6 (FL^{HD}) cells/fetus. For IUGT a 20 μ L suspension of GLOBE was administered IV via the vitelline peripheral yolk sac vessel at 5E+6 transforming units (TU)/fetus [31, 32]. We endeavoured to inject all fetuses in each litter. The maternal abdomen was closed in two layers with absorbable polyglactin sutures. Dams recovered in clean warm cages and were kept in a quiet environment until they

littered. Pups were cross-fostered on co-parturient CD1 dams and genotyped (Supplemental data). Postnatal transplantations were performed via tail-vein injections following IP busulfan and with CD26 inhibition of adult BM donor cells [25, 28]; some animals also received IV fludarabine (Supplemental data).

Surveillance and terminal harvest

Blood samples collected via tail venipuncture were analysed for haemoglobin concentration (Hb), RBC counts, red cell indices and haematocrit (Hct) on HemaVet 950 automated blood cell analyzer (Drew Scientific, FL). Blood smears were stained with May–Grunwald/Giemsa. B6-GFP chimerism was assessed in the peripheral blood MNC of HET and WT recipients for 24–32 weeks (w) of age by FACS for GFP and anti-mouse CD45.2 (conjugated to APC) and calculated as the percentage of GFP⁺ cells as a fraction of the total CD45.2 mononuclear cell population (all antibodies from Biolegend, Singapore). We analysed 50,000 to 100,000 events for FACS. Vector biodistribution was assessed with 15ng genomic DNA serially extracted from nucleated peripheral blood cells and from other tissues at terminal harvest [31], in a qPCR using standard late reverse transcript (LRT) primers specific for the responsive element (RRE) region complementary to the vector backbone (forward primer 5'-TGAAAGCGAAAGGGAAACCA-3', reverse primer 5'-CCGTGCGCGCTTCAG-3') with mouse *ACTB* as a loading control (forward primer 5'-GGTGCTAAGAAGGCTGTT-3' and reverse primer 5'-GGATACCTCTCTTGCTCTG-3') for estimation of average vector copy number (VCN) per cell.

Statistical Analysis

Parametric data are shown as mean \pm standard deviation (SD) and were analyzed using one-way or two-way analysis of variance and unpaired t tests. Non-parametric data were analyzed using the Mann–Whitney Test. $p \leq 0.05$ was considered significant. Analyses were performed on GraphPad Prism ver.6.04 (La Jolla, CA).

Results

IUHCT with adult BM and FL donor cells

To investigate the ability to rescue HbbTh3/+ and β^0/β^0 homozygotes we performed IP-IUHCT with coisogenic B6-GFP cells and compared outcomes with FL cells at the same doses (Figure 1A). There were no surviving homozygotes. BM^{HD} (n=10) demonstrated higher overall chimerism than BM^{LD} (n=14) over 20w ($1.3\pm0.9\%$ vs. $0.6\pm0.5\%$, $p=0.06$) with chimerism ranging from 0.2-3.1% and 0.01-1.2% respectively. Differences between both groups were significant at 8w (BM^{HD} 3.1 ± 2.5 vs. 0.7 ± 0.9 BM^{LD}, $p=0.001$, Figure 1B). HET mice (n=9) showed higher chimerism compared to WT (n=6) following BM^{LD} treatment ($1.6\pm0.4\%$ vs. 0.7 ± 0.2 , $p=0.05$); differences were significant at 20w (1.1% vs. 0.01% , $p=0.006$). HET maintained low chimerism of 0.9-4.1% until 24w while WT (initially 0.2-1.7%) lost engraftment by 20w (Figure 1C). As all surviving FL-IUHCT pups were WT, comparisons were made with BM-IUHCT WT mice injected in the same batch. FL^{LD} (n=8) demonstrated chimerism of 0.9-4.4% (mean $1.8\pm1.3\%$, undetectable at 32w) while FL^{HD} (n=8) showed 0.2-4.7% chimerism (mean $2.4\pm1.5\%$, $>1\%$ at 32w); both groups maintained stable low chimerism until ~28-32w (Figure 1D). Mean chimerism was similar between the groups ($2.4\pm1.5\%$ FL^{HD} vs. $1.8\pm1.2\%$ FL^{LD}, $p=0.45$). Higher chimerism resulted from FL^{HD} compared to BM^{HD} ($2.4\pm1.5\%$ vs. $1.2\pm0.5\%$, $p=0.07$); this difference was significant at 3w (4.3% vs. 0.6% , $p<0.05$). FL^{LD} showed similar overall chimerism to BM^{HD} ($1.8\pm1.3\%$ vs. $1.2\pm0.6\%$, $p=0.07$) and higher chimerism than BM^{LD} ($1.8\pm1.3\%$ vs. $0.7\pm0.5\%$, $p=0.03$).

Postnatal transplantation with busulfan and fludarabine

To counter early engraftment loss we boosted chimeric ($>1\%$) mice postnatally with B6-GFP adult BM cells with single and multiple doses. BM^{HD} and BM^{LD} IUHCT recipients were given $5E+6$ cells IV at 5w (Figure 2A). BM^{LD} produced chimerism of $2.0\pm0.9\%$ (range 0.7-3.2%) similar to BM^{HD} of $2.7\pm1.2\%$ (1.3-5.0%, $p=0.3$). Both lost chimerism after 16-20w. Unboosted BM^{HD} controls maintained $1.6\pm0.9\%$ chimerism; differences between BM^{HD} and controls were significant at 16w (1.3% vs. 0.3% , $p=0.04$). We performed multiple postnatal boosts to

maintain engraftment in BM^{LD} chimeras (>1%, n=8) and non-chimeras (n=8), using 5-30E+6 BM cells at 5, 10 and 15 postnatal weeks (Figure 2B). Post-boost chimerism was higher in chimeras compared to boosted non-chimeras (2.1 ± 0.9 vs 0.1 ± 0.1 , $p < 0.001$); individual time-point differences were significant at 24w (4.1 vs 0.001% , $p = 0.03$). Chimerism was maintained >1% and mostly >1.8% until 24 weeks. Non-IUHCT boosted mice (n=3) showed no postnatal chimerism. To assess the effect of transient immuno-suppression on sustained engraftment a preliminary assessment of fludarabine use was performed on BM^{LD} mice which showed <1% chimerism post-IUHCT (Figure 2C). Mice were given $10E+6$ BM cells at 4w and $8E+6$ cells at 6w (doses varied due to donor-cell availability). Fludarabine treatment (n=2) in addition to busulfan maintained chimerism at 0.2-0.7% after the second boost and resulted in higher donor-cell levels than busulfan alone (n=2, $0.4 \pm 0.2\%$ vs. $0.1 \pm 0.1\%$ respectively, $p = 0.3$). No differences in chimerism were observed between IUHCT mice with and without post-natal boost in this group. Non-IUHCT treated pups transplanted postnatally showed no chimerism.

We then boosted BM^{HD} mice with fludarabine and busulfan twice at 4w and 10w to determine engraftment duration, each dose being $10E+6$ cells (Figure 2D). IUHCT-treated chimeras (n=4) demonstrated $5.2 \pm 8.1\%$ donor-cell levels prior to postnatal boost which peaked at $15.1 \pm 25.1\%$ and decreased to $4.9 \pm 8.4\%$ after the first boost. The second produced a further increase to $9.6 \pm 16.2\%$ before eventual wastage to <1% by 24 weeks. Non-chimeras (n=4) did not show improvement after either boost, with levels remaining at 0.1-0.4%, significantly lower than chimeras ($0.2 \pm 0.1\%$ vs. 6.7 ± 4.6 , $p < 0.0001$, Figure 2D, inset). The highest expressers maintained levels of 2.3-52.4% and 1.0-14.7% and were HET by genotype. Both had smaller (though still enlarged) spleens compared to non-chimeras and untreated HET and ongoing haemolysis (Figure 2E,F). Treated HET showed increased MCV and reduced RDW compared with untreated HET (47.3 ± 6.2 fL vs. 35.4 ± 4.9 fL, and $34.3 \pm 4.3\%$ vs. $44.1 \pm 8.6\%$ respectively, $p < 0.05$), approaching the levels of WT (Figure 2G). Although treated HET still had lower Hb than WT there were no differences in MCV and MCH (49.9 ± 2.6 fL vs. 47.3 ± 6.2 fL and 16.2 ± 1.6 pg vs. 13.6 ± 2.9 pg respectively, $p > 0.05$).

IUGT with GLOBE

We generated GLOBE concentrates containing $\sim 2.6 \times 10^8$ TU/mL (Figure 3A). We injected 5×10^6 TU of GLOBE IV into hybrid fetuses at E15-16 to assess *in-vivo* IUGT (Figure 3B). Vector biodistribution and haematological indices in GLOBE-injected mice and controls were monitored from 3-24w postpartum. There was no difference in MNC VCN in peripheral blood between HET ($n=13$, 0.1 ± 0.2 copies/cell) and WT ($n=20$, 0.2 ± 0.1 copies/cell, $p=0.5$) detectable until 20w post-IUGT. Monthly VCN ranged from 0.001-0.4 copies/cell in both groups (Figure 3C). Terminal analyses at 20w ($n=3$) showed no VCN differences between treated HET and WT in blood (0.06 ± 0.05 vs. 0.18 ± 0.09 copies/cell), BM (0.01 ± 0.01 vs. 0.006 ± 0.003 copies/cell) and liver (0.001 ± 0.001 vs. 0.005 ± 0.004 copies/cell) respectively (Figure 3D). Most haematological indices were higher in treated than in untreated HET: Hb ($11.0 \pm 1.1\%$ vs. $9.3 \pm 1.4\%$, $p=0.04$), MCV (48.3 ± 3.5 fL vs. 35.4 ± 4.9 fL, $p=0.004$) and RBC ($7.7 \pm 0.9 \times 10^6/\mu\text{L}$ vs. $5.3 \pm 0.6 \times 10^6/\mu\text{L}$, $p=0.001$), with a trend towards normal RDW ($35.8 \pm 1.6\%$ vs. $44.1 \pm 8.6\%$, $p=0.1$). Significant time-point differences between treated and untreated HET are indicated in Figure 3E. Haematological values remained statistically lower in treated HET compared to WT.

Discussion

We demonstrate partial improvement in the HbbTh3/+ mouse model with two clinically-applicable strategies of low-dose in-vivo GLOBE-IUGT and IUHCT with postnatal boost with fludarabine, which achieved preferential engraftment in chimeras and heterozygotes over non-chimeras and wild-type offspring. While we did not achieve full phenotypic correction with either, we observed that most haematological indices in treated HET improved compared to non-treated HET, an effect marginally superior with IUGT despite the low VCN. IUHCT has long been limited by low-level chimerism in animal models due to steadfast engraftment barriers [33, 34], which have been challenged through manipulation of donor cells and recipient BM microenvironment.

We performed IP-IUHCT with a dose of 2.5×10^6 cells/fetus, similar to studies which have demonstrated dose-dependent chimerism of 0-7.5% (2×10^4 - 2×10^6 cells/fetus IP), 10-20% (2.5×10^6 cells/fetus IH) and 5-20% (5×10^6 cells/fetus IP) with eventual long-term decline in WT mice [14, 35, 36], and stable chimerism of 0.5-1.7% in the CLAD canine model (IP, 2.5×10^8 CD34+ cells/kg) [37]. In thalassaemia and SCD mice however, single allogeneic IP-IUHCT of 5×10^6 adult BM cells/pup resulted in lower chimerism of 1.1-4.1% over 10 months [33, 38]. These data are informative of the expected chimerism resulting from a starting IP/IH dose of 2.5×10^6 cells of ~5-20% in WT mice and ~1-4% in haemoglobinopathy models. Our donor-cell chimerism of 2.3-4.9% at 3 postnatal weeks is comparable but the duration of engraftment was much shorter without postnatal boost. We administered $\sim 4 \times 10^9$ cells/kg at E14-16 (weights 0.5-0.7g), two log-folds higher than the recommended dose for optimal engraftment in postnatal umbilical cord blood (UCB) transplantations of $\sim 4 \times 10^7$ nucleated cells/kg, albeit with conditioning therapy [39]. The engraftment shortfall in our model reflects other physiological such as the lack of space and immune rejection, and technical barriers. Although IV injections allow delivery of a much higher cell dose ($20\text{-}30 \times 10^6$ vs. 5×10^6 IP) with significantly higher short-term engraftment [40], there is little difference in long-term

engraftment between IV-IUHCT (20E+6 cells/fetus, 5-10% at 6 months [28, 40]) and IH or IP-IUHCT (2.5-5E+6 cells/fetus, 5-20% [14, 35, 36]). IV-IUHCT at this early gestation is technically demanding and IP or IH injections are more reproducible, an important consideration for clinical application particularly when fetuses are targeted in early gestation [41]. Congenic IUHCT produces lower, but ultimately more stable, chimerism compared to the allogenic IUHCT in which the majority of engrafted cells are lost through immunological clearance [13, 35]. Our congenic transplantation showed rapid engraftment loss over 24 weeks following initial chimerism of 2.3-4.9% at a comparable dose of 5E+6 cells/fetus; this shortfall in long-term stability is probably related to other intrinsic barriers in this disease model. Human FL cells have demonstrated distinct competitive advantages over adult BM and UCB HSC in transplantation, and a postnatal dose of 1E+6 resulted in partial erythropoietic correction in a neonatal thalassaemic murine model [42, 43]. The chimerism of 1.8-2.4% we attained with FL-IUHCT were at the lower range reported by Hayashi et al of 2-10% with 1E+6 FL cells/fetus which improved haematological indices transiently [33]. There are practical and ethical challenges of using FL in the presence of suitable alternatives such as maternal BM and UCB HSC [17, 44]. FL-IUHCT in human recipients and large animals has, in contrast to murine and ovine studies [33, 45], consistently produced low and transient chimerism [11, 15], and similarly poor engraftment in postnatal recipients [46]. CD34 harvest from FL or UCB is limited, although the typical yield will be sufficient for fetal transplantation [47]. Maternal BM is readily available for repeat transplantations and produced 22% chimerism following intracardiac delivery of 2.5-5.2E+10 cells/kg at 0.67G in canines [17], though our own experience with maternal BM-IUHCT in macaques using ~1E+9 cells/kg in early gestation has produced low macro- and microchimerism (manuscript in preparation).

Transfusion independence in successfully transplanted patients with thalassaemia requires 10-20% engraftment of normal HSC, facilitated by aggressive myeloablation aimed at maximal reduction of BM cells, as persistent engraftment depends heavily on minimising residual host cells [48, 49]. With IUHCT in the thalassaemic mouse, without the benefit of BM

clearance a single dose of $5E+6$ adult BM donor cells/fetus resulted in short-term chimerism in our study (2.3-4.9%) similar to other β -thalassaemia murine studies (1.1-4.1%) using the same dose [33, 38]. Chronic changes in the BM microenvironment impair the capacity of transduced HSC to engraft and mature in the long-term [16]. The thalassaemic niche is under constant physiological stress from ineffective erythropoiesis and compensatory expansion of erythroid progenitors; osteoporosis and osteopenia may add further strain [50]. These events alter the macromolecular structure and biochemical content of BM cells affecting interactions between HSC and other BM cells which may contribute to lower engraftment efficacy [51, 52]. IUHCT-treated β -thalassaemia mice illustrate this with five-fold lower chimerism than WT pups treated with the same donor-cell dose [33, 36, 38], as does IUHCT in human fetuses prenatally-diagnosed with major haemoglobinopathies [15].

Thus IUHCT may be most useful as part of a multi-pronged approach to induce donor-specific tolerance in-utero prior to postnatal transplantation to maintain therapeutic engraftment. These combined approaches have employed booster doses of $30E+6$ cells/pup which resulted in $>1\%$ chimerism mainly due to the competitive advantage conferred by BM clearance or CD26 inhibition preceding transplantation [25, 28, 38, 53]. High-dose total body irradiation (TBI) improved the initial $\sim 2\%$ chimerism in thalassaemic mice to $\sim 70\%$ (vs. $\sim 15\%$ without TBI), sufficient to correct splenomegaly and erythropoiesis [38, 53]. Enhanced postnatal engraftment was similarly achieved with CD26 inhibition or pre-transplantation high-dose busulfan [25, 28]. We used IP-IUHCT of $5E+6$ cells/fetus to create donor-cell tolerance, followed by busulfan and fludarabine to create space and maintain peripheral tolerance to postnatal boost. Fludarabine is well tolerated when combined with busulfan in reduced-toxicity conditioning regimens for allogeneic HSCT for a range of haematological diseases [54]. We also used a much lower booster dose of $10E+6$ cells/pup at 4 weeks ($\sim 20g$) and would have delivered $5E+8$ cells/kg, similar to the optimal nucleated-cell dose for postnatal BM transplantation in juveniles of $4E+8$ cells/kg [55]. We were unable to consistently provide sufficient donor cells for doses of $30E+6$ cells/pup. Thus we increased

postnatal immune-suppression while keeping the booster dose low to determine our ability to maintain chimerism >1.8%, the threshold associated with sustained engraftment and tolerance in mice [14]. We observed a lower chimerism of 3-20% among boosted chimeric animals. Treated HET still showed improved MCV and RDW and smaller spleens compared to untreated HET; though Hb was still lower than WT mice, the differences in MCV and MCH between treated HET and WT mice were now insignificant, similar to other studies [33]. As perinatal TBI carries significant toxicity [56], interventions to overcome host competition have been reviewed including early high-dose transplantation before endogenous BM population [17] and host BM clearance with anti-c-kit receptor antibodies [57]. However, to achieve IUHCT before BM population in a human fetus, prenatal diagnosis of a major haemoglobinopathy should be completed before 16 weeks' gestation as fetal BM erythropoiesis begins at 16-18 weeks (0.4-0.45G) [58]. While possible, we anticipate that the majority of at-risk patients will diagnosed after this gestation and some degree of BM clearance will still be necessary to boost engraftment. Alternatively, intrauterine manipulation of the fetal BM may disrupt its stability and increase the risk of teratogenicity.

GLOBE-IUGT significantly improved haematological indices in treated HET mice, though with incomplete phenotype correction. This is the second demonstration of a therapeutic effect with direct LV-IUGT in thalassaemic mice [20]. Ex-vivo gene transfer (GT) in juveniles and adults with β -haemoglobinopathies demonstrate varying degrees of success from modest haemoglobin improvement to complete transfusion-independence [21]. Haemopoietin correction and oncogenic risk both increase with higher VCN [59-61]. Erythropoietic correction is anticipated with LV-mediated HSC transduction of ~10-20% and severe murine β -thalassemia intermedia was cured with a VCN of 1-2.5 LV copies/HSC [59-61]. We used a LV dose of $5E+6$ TU/mL, 1-2 log-folds lower than doses in published studies, to keep VCN to <1 copy/nucleated blood cell which carries the lowest risk of integration mutagenesis [60-62]. At this low VCN we still observed a significant improvement in Hb, MCV and RBC counts and a trend towards a lower RDW in treated compared to untreated

HET. Although these values were significantly different from WT mice, they suggest the utility of in-vivo LV-IUGT once dosage is optimised. Improved design and enhanced therapeutic efficacy expand the utility of LV-mediated HSC transduction for affected individuals for whom curative HSCT is unavailable or prohibitively expensive [63]. Of particular concern is the increased integration potential near growth-control regions due to the open structure of fetal chromatin [64]. GLOBE has a low capacity for genotoxicity because of low-frequency integration, transgene expression restricted to differentiated erythropoietic cells, and low incidence of aberrant gene-splicing in human cell lines [24, 61], and this profile is unlikely to change with in-utero administration.

While both direct LV-IUGT and IUHCT are potential options for fetal therapy, consensus opinion is that IUHCT is more suitable for clinical trials presently due to unanswered questions regarding IUGT safety; however recent promising clinical trials in children may soon change this perspective [65, 66]. It may be argued that intrauterine therapy should be reserved for historically lethal α -thalassaemia major; with the considerable limitations of conventional therapy, fetal intervention will still benefit the chronic β -haemoglobinopathies. IUHCT will be valuable in a multi-pronged strategy aimed at donor-cell tolerance through fetal chimerism and enhanced engraftment through postnatal therapy.

TOTAL WORD COUNT: 3812

Author contributions

NMD and KWT produced lentiviral vector, designed and executed animal and molecular experiments and analysed data. LGT, SSWS, SMKB, DS, NJ, YWT performed animal and molecular experiments and analysed data. AB and MC contributed to experimental design. SNW, MNA, JKYC and CNZM designed and performed experiments, provided vector plasmids (MNA), performed data analysis and wrote and revised the manuscript.

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References

- [1] Weatherall DJ. The challenge of haemoglobinopathies in resource-poor countries. *Br J Haematol.* 2011;154:736-744.
- [2] Kreger EM, Singer ST, Witt RG, et al. Favorable outcomes after in utero transfusion in fetuses with alpha thalassemia major: a case series and review of the literature. *Prenat Diagn.* 2016;36:1242-1249.
- [3] Piel FB, Hay SI, Gupta S, Weatherall DJ, Williams TN. Global burden of sickle cell anaemia in children under five, 2010-2050: modelling based on demographics, excess mortality, and interventions. *PLoS medicine.* 2013;10:e1001484.
- [4] Maakaron JE, Cappellini MD, Taher AT. An update on thalassemia intermedia. *J Med Liban.* 2013;61:175-182.
- [5] Baronciani D, Angelucci E, Potschger U, et al. Hemopoietic stem cell transplantation in thalassemia: a report from the European Society for Blood and Bone Marrow Transplantation Hemoglobinopathy Registry, 2000-2010. *Bone Marrow Transplant.* 2016;51:536-541.
- [6] Haub C. Fact Sheet: World Population Trends 2012. Population Reference Bureau; 2012.
- [7] Gaziev J, Isgro A, Sodani P, et al. Optimal Outcomes in Young Class 3 Patients With Thalassemia Undergoing HLA-Identical Sibling Bone Marrow Transplantation. *Transplantation.* 2016;100:925-932.
- [8] Thrasher AJ, Hacein-Bey-Abina S, Gaspar HB, et al. Failure of SCID-X1 gene therapy in older patients. *Blood.* 2005;105:4255-4257.
- [9] Mattar CN, Choolani M, Biswas A, Waddington SN, Chan JK. Fetal gene therapy: recent advances and current challenges. *Expert Opin Biol Ther.* 2011;11:1257-1271.
- [10] McClain LE, Flake AW. In utero stem cell transplantation and gene therapy: Recent progress and the potential for clinical application. *Best practice & research Clinical obstetrics & gynaecology.* 2016;31:88-98.
- [11] Mattar CN, Biswas A, Choolani M, Chan JK. The case for intrauterine stem cell transplantation. *Best practice & research Clinical obstetrics & gynaecology.* 2012;26:683-695.
- [12] Gotherstrom C, Westgren M, Shaw SW, et al. Pre- and postnatal transplantation of fetal mesenchymal stem cells in osteogenesis imperfecta: a two-center experience. *Stem Cells Transl Med.* 2014;3:255-264.
- [13] Peranteau WH, Endo M, Adibe OO, Flake AW. Evidence for an immune barrier after in utero hematopoietic-cell transplantation. *Blood.* 2007;109:1331-1333.
- [14] Durkin ET, Jones KA, Rajesh D, Shaaban AF. Early chimerism threshold predicts sustained engraftment and NK-cell tolerance in prenatal allogeneic chimeras. *Blood.* 2008;112:5245-5253.
- [15] Tiblad E, Westgren M. Fetal stem-cell transplantation. *Best Practice & Research Clinical Obstetrics & Gynaecology.* 2008;22:189-201

- [16] Cavazzana M, Ribeil JA, Lagresle-Peyrou C, Andre-Schmutz I. Hematopoietic stem cell gene therapy: the point of view of the diseased bone marrow. *Stem cells and development*. 2016.
- [17] Vreacenak JD, Pearson EG, Santore MT, et al. Stable long-term mixed chimerism achieved in a canine model of allogeneic in utero hematopoietic cell transplantation. *Blood*. 2014;124:1987-1995.
- [18] Petersen SM, Gendelman M, Murphy KM, et al. Use of T-cell antibodies for donor dosaging in a canine model of in utero hematopoietic stem cell transplantation. *Fetal diagnosis and therapy*. 2007;22:175-179.
- [19] Petersen SM, Gendelman M, Murphy KM, et al. In utero hematopoietic stem cell transplantation in canines: exploring the gestational age window of opportunity to maximize engraftment. *Fetal diagnosis and therapy*. 2013;33:116-121.
- [20] Han XD, Lin C, Chang J, Sadelain M, Kan YW. Fetal gene therapy of alpha-thalassemia in a mouse model. *Proc Natl Acad Sci U S A*. 2007;104:9007-9011.
- [21] Malik P. Gene Therapy for Hemoglobinopathies: Tremendous Successes and Remaining Caveats. *Mol Ther*. 2016;24:668-670.
- [22] Cicalese MP, Aiuti A. Clinical applications of gene therapy for primary immunodeficiencies. *Hum Gene Ther*. 2015;26:210-219.
- [23] Yang B, Kirby S, Lewis J, Detloff PJ, Maeda N, Smithies O. A mouse model for beta 0-thalassemia. *Proc Natl Acad Sci U S A*. 1995;92:11608-11612.
- [24] Miccio A, Cesari R, Lotti F, et al. In vivo selection of genetically modified erythroblastic progenitors leads to long-term correction of beta-thalassemia. *Proc Natl Acad Sci U S A*. 2008;105:10547-10552.
- [25] Ashizuka S, Peranteau WH, Hayashi S, Flake AW. Busulfan-conditioned bone marrow transplantation results in high-level allogeneic chimerism in mice made tolerant by in utero hematopoietic cell transplantation. *Exp Hematol*. 2006;34:359-368.
- [26] Quesenberry PJ, Goldberg LR, Dooner MS. Concise reviews: A stem cell apostasy: a tale of four H words. *Stem cells (Dayton, Ohio)*. 2015;33:15-20.
- [27] Merianos DJ, Tiblad E, Santore MT, et al. Maternal alloantibodies induce a postnatal immune response that limits engraftment following in utero hematopoietic cell transplantation in mice. *J Clin Invest*. 2009;119:2590-2600.
- [28] Peranteau WH, Endo M, Adibe OO, Merchant A, Zoltick PW, Flake AW. CD26 inhibition enhances allogeneic donor-cell homing and engraftment after in utero hematopoietic-cell transplantation. *Blood*. 2006;108:4268-4274.
- [29] Antoniou M, Geraghty F, Hurst J, Grosveld F. Efficient 3'-end formation of human beta-globin mRNA in vivo requires sequences within the last intron but occurs independently of the splicing reaction. *Nucleic Acids Res*. 1998;26:721-729.
- [30] Dighe N, Khoury M, Mattar C, et al. Long-term reproducible expression in human fetal liver hematopoietic stem cells with a UCOE-based lentiviral vector. *PLoS One*. 2014;9:e104805.

- [31] Waddington SN, Mitrophanous KA, Ellard F, et al. Long-term transgene expression by administration of a lentivirus-based vector to the fetal circulation of immuno-competent mice. *Gene Ther.* 2003;10:1234-1240.
- [32] Waddington SN, Buckley SMK, Nivsarkar M, et al. In utero gene transfer of human factor IX to fetal mice can induce postnatal tolerance of the exogenous clotting factor. *Blood.* 2003;101:1359-1366.
- [33] Hayashi S, Abdulmalik O, Peranteau WH, et al. Mixed chimerism following in utero hematopoietic stem cell transplantation in murine models of hemoglobinopathy. *Exp Hematol.* 2003;31:176-184.
- [34] Shaaban AF, Kim HB, Gaur L, Liechty KW, Flake AW. Prenatal transplantation of cytokine-stimulated marrow improves early chimerism in a resistant strain combination but results in poor long-term engraftment. *Exp Hematol.* 2006;34:1278-1287.
- [35] Nijagal A, Wegorzewska M, Jarvis E, Le T, Tang Q, MacKenzie TC. Maternal T cells limit engraftment after in utero hematopoietic cell transplantation in mice. *J Clin Invest.* 2011;121:582-592.
- [36] Hayashi S, Peranteau WH, Shaaban AF, Flake AW. Complete allogeneic hematopoietic chimerism achieved by a combined strategy of in utero hematopoietic stem cell transplantation and postnatal donor lymphocyte infusion. *Blood.* 2002;100:804-812.
- [37] Peranteau WH, Heaton TE, Gu YC, et al. Haploidentical in utero hematopoietic cell transplantation improves phenotype and can induce tolerance for postnatal same-donor transplants in the canine leukocyte adhesion deficiency model. *Biol Blood Marrow Transplant.* 2009;15:293-305.
- [38] Peranteau WH, Hayashi S, Abdulmalik O, et al. Correction of murine hemoglobinopathies by prenatal tolerance induction and postnatal nonmyeloablative allogeneic BM transplants. *Blood.* 2015;126:1245-1254.
- [39] Gluckman E, Rocha V, Boyer-Chammard A, et al. Outcome of cord-blood transplantation from related and unrelated donors. Eurocord Transplant Group and the European Blood and Marrow Transplantation Group. *N Engl J Med.* 1997;337:373-381.
- [40] Boelig MM, Kim AG, Stratigis JD, et al. The Intravenous Route of Injection Optimizes Engraftment and Survival in the Murine Model of In Utero Hematopoietic Cell Transplantation. *Biol Blood Marrow Transplant.* 2016.
- [41] Fox C, Martin W, Somerset DA, Thompson PJ, Kilby MD. Early intraperitoneal transfusion and adjuvant maternal immunoglobulin therapy in the treatment of severe red cell alloimmunization prior to fetal intravascular transfusion. *Fetal diagnosis and therapy.* 2008;23:159-163.
- [42] Bethel CAI, Muragesh D, Harrison MR, Mohandas N, Rubin ER. Fetal Hematopoietic Stem Cell Transplantation Into Beta Thalassemic Mice *Journal of pediatric surgery.* 1993;28 1232-1238.
- [43] Taylor PA, McElmurry RT, Lees CJ, Harrison DE, Blazar BR. Allogenic fetal liver cells have a distinct competitive engraftment advantage over adult bone marrow cells when infused into fetal as compared with adult severe combined immunodeficient recipients. *Blood.* 2002;99:1870-1872.

- [44] Lepus CM, Gibson TF, Gerber SA, et al. Comparison of human fetal liver, umbilical cord blood, and adult blood hematopoietic stem cell engraftment in NOD-scid/gammac^{-/-}, Balb/c-Rag1^{-/-}gammac^{-/-}, and C.B-17-scid/bg immunodeficient mice. *Hum Immunol*. 2009;70:790-802.
- [45] Flake AW, Hendrick MH, Rice HE, Tavassoli M, Zanjani ED. Enhancement of human hematopoiesis by mast cell growth factor in human-sheep chimeras created by the in utero transplantation of human fetal hematopoietic cells. *Exp Hematol*. 1995;23:252-257.
- [46] Ishii T, Eto K. Fetal stem cell transplantation: Past, present, and future. *World Journal of Stem Cells*. 2014;6:404-420.
- [47] Gluckman E. Fetal and neonatal hemopoietic stem cells: considerations in transplantation. *Nouvelle revue francaise d'hematologie*. 1990;32:421.
- [48] Persons DA. The challenge of obtaining therapeutic levels of genetically modified hematopoietic stem cells in beta-thalassemia patients. *Ann N Y Acad Sci*. 2010;1202:69-74.
- [49] Andreani M, Nesci S, Lucarelli G, et al. Long-term survival of ex-thalassemic patients with persistent mixed chimerism after bone marrow transplantation. *Bone Marrow Transplantation*. 2000;25:401.
- [50] Leung TF, Hung EC, Lam CW, et al. Bone mineral density in children with thalassaemia major: determining factors and effects of bone marrow transplantation. *Bone Marrow Transplant*. 2005;36:331-336.
- [51] Aksoy C, Guliyev A, Kilic E, Uckan D, Severcan F. Bone Marrow Mesenchymal Stem Cells in Patients with Beta Thalassemia Major: Molecular Analysis with Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy Study as a Novel Method. *Stem cells and development*. 2012;21:2000-2011.
- [52] Rosen CJ, Ackert-Bicknell C, Rodriguez JP, Pino AM. Marrow Fat and the Bone Microenvironment: Developmental, Functional, and Pathological Implications. *Critical reviews in eukaryotic gene expression*. 2009;19:109-124.
- [53] Peranteau WH, Hayashi S, Hsieh M, Shaaban AF, Flake AW. High-level allogeneic chimerism achieved by prenatal tolerance induction and postnatal nonmyeloablative bone marrow transplantation. *Blood*. 2002;100:2225-2234.
- [54] Anurathapan U, Pakakasama S, Rujkijyanont P, et al. Pretransplant immunosuppression followed by reduced-toxicity conditioning and stem cell transplantation in high-risk thalassemia: a safe approach to disease control. *Biol Blood Marrow Transplant*. 2013;19:1259-1262.
- [55] Rocha V, Garnier F, Ionescu I, et al. Hematopoietic stem-cell transplantation using umbilical-cord blood cells. *Rev Invest Clin*. 2005;57:314-323.
- [56] Wilde G, Sjostrand J. A clinical study of radiation cataract formation in adult life following gamma irradiation of the lens in early childhood. *Br J Ophthalmol*. 1997;81:261-266.
- [57] Witt RG, Kreger EM, Buckman LB, et al. Systemic multilineage engraftment in mice after in utero transplantation with human hematopoietic stem cells. *Blood Adv*. 2018;2:69-74.
- [58] Forestier F, Daffos F, Catherine N, Renard M, Andreux JP. Developmental hematopoiesis in normal human fetal blood. *Blood*. 1991;77:2360-2363.

- [59] Miccio A, Cesari R, Lotti F, et al. In vivo selection of genetically modified erythroblastic progenitors leads to long-term correction of beta-thalassemia. *Proc Natl Acad Sci U S A*. 2008;105:10547-10552.
- [60] Persons DA, Hargrove PW, Allay ER, Hanawa H, Nienhuis AW. The degree of phenotypic correction of murine beta -thalassemia intermedia following lentiviral-mediated transfer of a human gamma-globin gene is influenced by chromosomal position effects and vector copy number. *Blood*. 2003;101:2175-2183.
- [61] Roselli EA, Mezzadra R, Frittoli MC, et al. Correction of beta-thalassemia major by gene transfer in haematopoietic progenitors of pediatric patients. *EMBO Mol Med*. 2010;2:315-328.
- [62] Zhou S, Fatima S, Ma Z, et al. Evaluating the Safety of Retroviral Vectors Based on Insertional Oncogene Activation and Blocked Differentiation in Cultured Thymocytes. *Molecular Therapy*. 2016;24:1090-1099.
- [63] Ferrari G, Cavazzana M, Mavilio F. Gene Therapy Approaches to Hemoglobinopathies. *Hematol Oncol Clin North Am*. 2017;31:835-852.
- [64] Manesia JK, Franch M, Tabas-Madrid D, et al. Distinct molecular signature of murine fetal liver and adult hematopoietic stem cells identifies novel regulators of hematopoietic stem cell function. *Stem cells and development*. 2016.
- [65] MacKenzie TC, David AL, Flake AW, Almeida-Porada G. Consensus statement from the first international conference for in utero stem cell transplantation and gene therapy. *Front Pharmacol*. 2015;6:15.
- [66] Mendell JR, Al-Zaidy S, Shell R, et al. Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy. *N Engl J Med*. 2017;377:1713-1722.

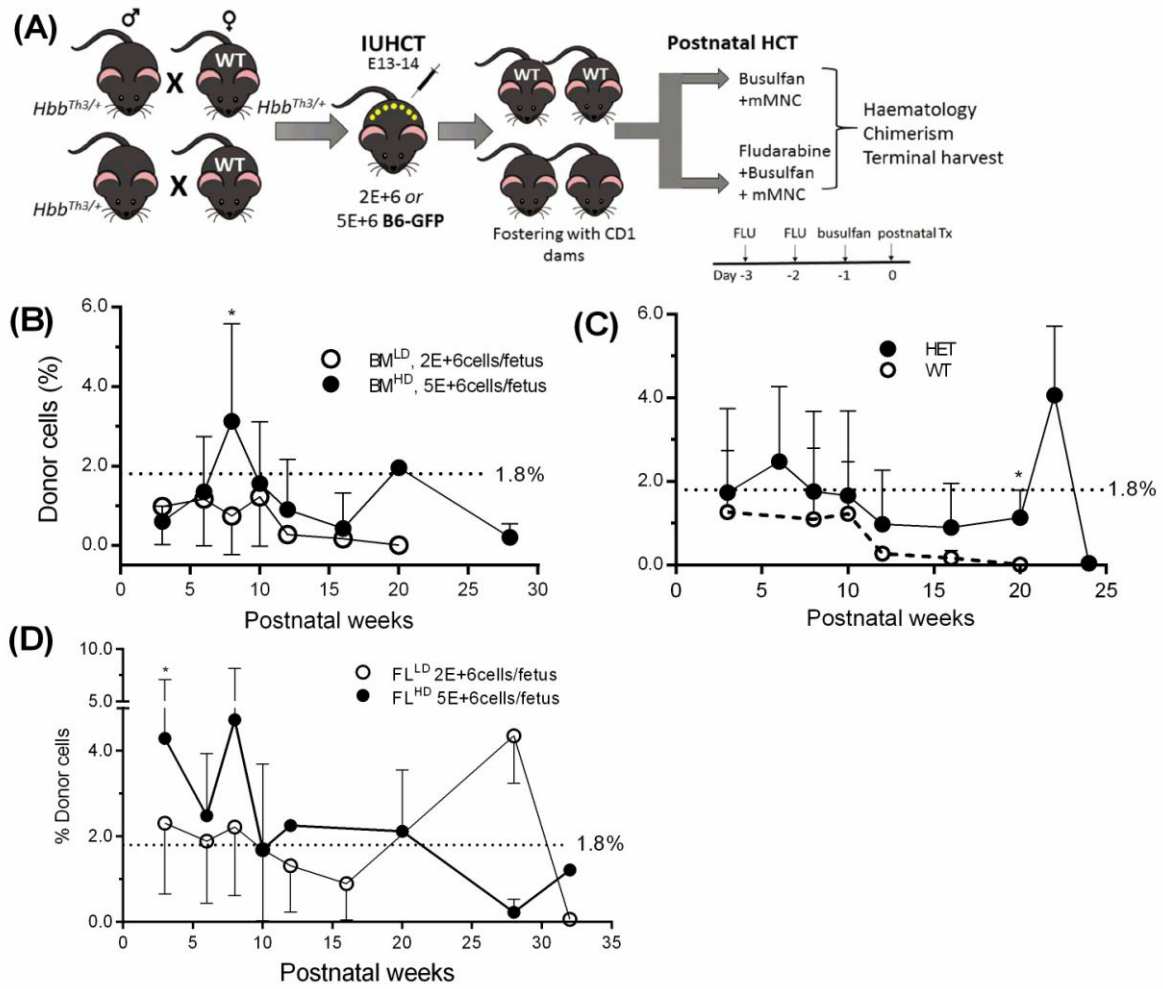
Figure Legends

Figure 1. Murine adult bone marrow (BM) and fetal liver (FL) mononuclear cells transplanted into HbbTh3 murine fetuses. (A) BM^{LD} (2E+6) or BM^{HD} (5E+6) B6-GFP donor cells were administered to E13-14 pups (HbbTh3/+ males x HbbTh3/+ or B6 females). (B) This produced overall chimerism of 0.6±0.5% vs. 1.3±0.9% respectively (p=0.06), significant at 8 weeks (0.7±0.9% vs. 3.1±2.5%, p=0.001). (C) HET mice had higher chimerism compared to WT after BM^{LD} treatment (1.6±0.4% vs. 0.7±0.2, p=0.05) and maintained low chimerism of 0.9-4.1% until 24 weeks while WT chimerism (0.2-1.7%) was lost by 20 weeks (D) FL^{LD} demonstrated 0.9-4.4% chimerism until 32 weeks; FL^{HD} showed 0.2-4.7% chimerism (>1% at 32w). Mean chimerism was similar between the groups (2.4±1.5% FL^{HD} vs. 1.8±1.2% FL^{LD}, p=0.45). There were no surviving HET FL-IUHCT pups. *p<0.05.

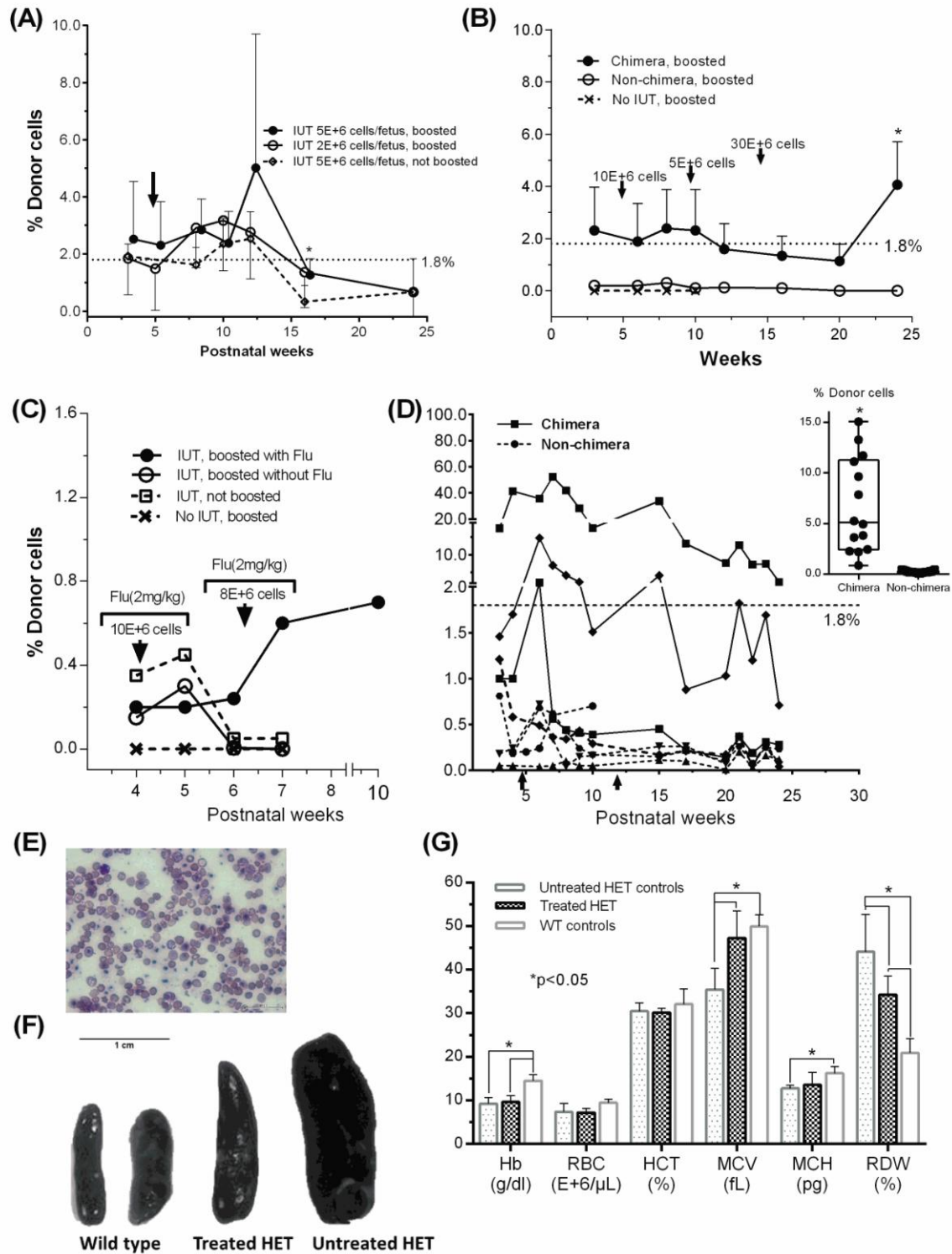
Figure 2. Postnatal transplantation to boost chimerism following intrauterine transplantation. Adult BM mononuclear cells were postnatally transplanted into chimeric and non-chimeric IUHCT recipients to boost engraftment. Black arrows indicate time-points of postnatal transplantation. (A) BM^{HD} and BM^{LD} IUHCT recipients were given 5E+6 cells IV at 5 weeks. BM^{LD} produced 2.0±0.9% chimerism (range 0.7-3.2%) and BM^{HD} produced 2.7±1.2% chimerism (1.3-5.0%, p=0.3). Both lost chimerism after 16-20 weeks. Unboosted BM^{HD} controls maintained 1.6±0.9% chimerism. (B) Multiple boosts to BM^{LD} chimeras and non-chimeras with 5-30E+6 BM cells produced higher donor-cell levels in chimeras (2.1±0.9 vs 0.1±0.1, p<0.001) which was maintained mostly >1.8% until 24 weeks. Non-IUHCT boosted mice (n=3) showed no postnatal chimerism. (C) Transient immuno-suppression was produced with IV fludarabine in BM^{LD} recipients (<1% chimerism post-IUHCT) before postnatal boosts of 10E+6 BM cells at 4 weeks and 8E+6 cells at 6 weeks. Fludarabine-treated mice maintained chimerism at 0.2-0.7% after the second boost and had higher donor-cell levels than non-fludarabine treated controls (0.4±0.2% vs. 0.1±0.1% respectively, p=0.3). IUHCT mice with and without post-natal boost and non-IUHCT treated pups transplanted postnatally showed minimal or no chimerism. (D) BM^{HD} mice were treated with fludarabine and 10E+6 cells twice at 4 and 10 weeks. Chimeras showed increased donor-cell levels after each boost (overall chimerism 6.7±4.6%) before eventual wastage to <1% by 24 weeks. Non-chimeras did not show improvement after either boost (inset). Two treated chimeras maintained levels of 2.3-52.4% and 1.0-14.7% and were HET by genotype. Both had ongoing haemolysis (E) and smaller (though still enlarged) spleens (F) compared to non-chimeras. (G) Treated HET showed increased MCV and reduced RDW compared with untreated HET (47.3±6.2fL vs. 35.4±4.9fL, and 34.3±4.3% vs. 44.1±8.6% respectively, p<0.05), approaching the levels of WT. Treated HET had lower Hb than WT but there were no differences in MCV and MCH (49.9±2.6fL vs. 47.3±6.2fL and 16.2±1.6pg vs. 13.6±2.9pg respectively, p>0.05). *p<0.05.

Figure 3: Intrauterine injection of lentiviral vectors expressing murine B globin in HbbTh3 murine fetuses. To examine the effectiveness of IUGT we administered i.v. injections of GLOBE (A), a lentiviral vector constructed to express the human β -globin mini-gene in the erythropoietic lineage, driven by the β -locus control region (β -LCR, comprising HS2/HS3). (B) Pups were injected at E15-16 with a LV dose of 5E+6 TU in 20 μ L via the perivitelline vein. (C) VCN in treated HET and WT mice were similar all time-points (means 0.1 and 0.2 copies/cell respectively, p=0.5); both groups demonstrated an increasing VCN that peaked at 8 weeks (mean ~0.4 ±1.4 copies/cell) and was not detectable (ND) by 24 weeks. (D) There were no differences in low-level VCN in blood, BM and liver at 20 weeks. (E) Haematological parameters showed an overall improvement in treated vs. untreated HET: Hb (11.0±1.1g% vs. 9.3±1.4g%, p=0.04), MCV (48.3±3.5fL vs. 35.4±4.9fL, p=0.004) and RBC (7.7±0.9x10⁶/ μ L vs. 5.3±0.6 x10⁶/ μ L, p=0.001) with reduced RDW (35.8±1.6% treated vs. 44.1±8.6% untreated, p=0.1). *p<0.05.

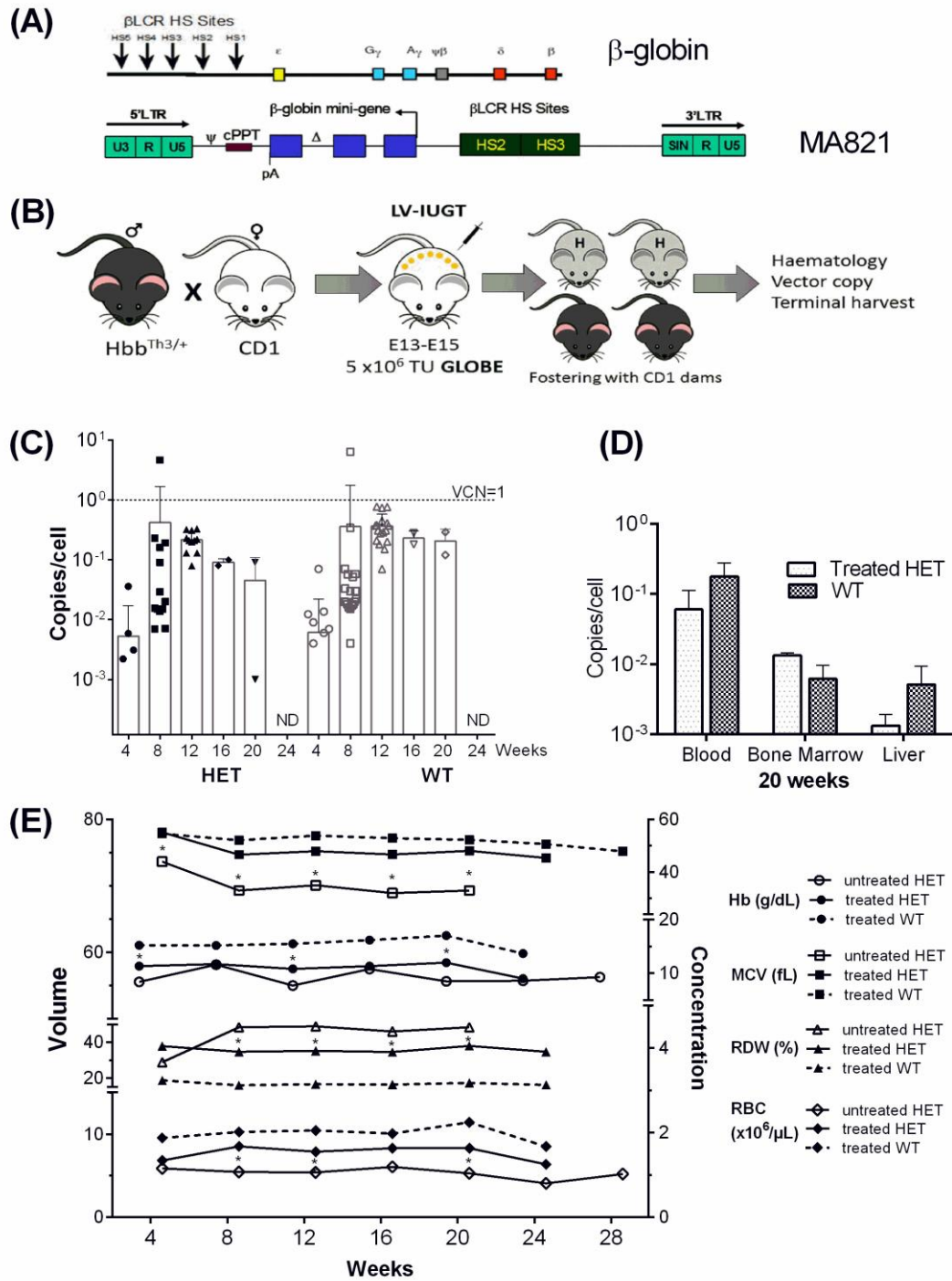
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EXPHEM R2 FIGURE 1.tif



EXPHEM R2 FIGURE 2.tif



EXPHEM R2 FIGURE 3.tif